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PRODUCTION OF LACTIC ACID FROM CHEESE WHEY BY IMMOBILIZED CELL REACTOR OF *STRAIN LACTOCOCCUS LACTIS SUBSP LACTIS SP1* ADSORBED ONTO POZZOLANA BED

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ABSTRACT

The technique of cell immobilization biomass using porous support particles, which is attractive from the point of view of simplicity and convenience, relies on the inherent ability of adhesive cells as a consequence of their growth to form films around and in the support material. In the present study immobilized cell reactor of the strain *Lactococcus lactis subsp Lactis SP1* naturally adsorbed onto pozzolana beds was constructed and used for the investigation of batch and continuous production of L+ lactic acid from nutritionally enriched whey. High final L+ lactic acid concentration was achieved; 15,57 g/l corresponding at 77% of lactose consumption, in batch fermentation filled with 6,25 particle size of pozzolana. The best results were obtained when the immobilized cells bioreactor was operated in a continuous mode and both dilutions rates were manipulated. At Dilution rate 0,46 h^{-1} a concentration of 21,8 g/:l of lactic acid was achieved in reactor effluent with a conversion yield of 88,8 % and volumetric reactor productivity of 30,57 $g.l^{-1}h^{-1}$

KEYWORDS: L+ Lactic Acid, Immobilized Cell Reactor, *Lactococcus lactis subsp lactis.* Pozzolana Bed, Batch Fermentation, Continuous Fermentation, Biofilm

NOMENCLATURE

XRD	**:**	X ray diffraction
SEM	**:**	Surface electronic microscopy
WS	**:**	Whey supplemented
PZ	**:**	Pozzolana
MATS	**:**	Microbial adhesion to solvents

INTRODUCTION

Lactic acid is now considered to be one of the most useful chemicals, it has a wide range of applications in the food, pharmaceutical, cosmetic, textiles and chemicals industries (Young et *al.,* 2006; Panesar et *al.,* 2010), and recently its potential for producing biocompatible and biodegradable plastics has been actively pursued (Majid J. et *al.,* 2010; Illmen et *al.,* 2007). Compared to chemical synthesis, microbial fermentation is a better alternative because it leads to the production of optically pure lactic acid and offers advantages in the utilization of renewable carbohydrates (Illmen et *al.,* 2007). Lactic acid can obtain from fermentation processes by using lactic acid bacteria. Although batch processes are currently used, a number of more advanced techniques have been investigated in order to improve the process efficiency. There is an increasing interest in the practical applications of immobilized microbial cell systems for

the production a view to improve the reactor productivity (Datta et *al*., 1995). The concept of cell immobilization provides a promising strategy for the use of cells in a bioreactor for easy scale up and industrial production (Goncalves et *al*., 1992; Mirdamadi et *al*., 2008). Interest in natural cell immobilization or biofilms for lactic acid fermentation has developed considerably over the last few decades. Many studies report the benefits associated with biofilms as industrial methods for food production and for wastewater treatment, since the formation represents a protective means of microbial growth offering survival advantages to cells in toxic environments. The formation of biofilms is a natural process in which microbial cells adsorb to a support without chemicals or polymers that entrap the cells and is dependent on the reactor environment, microorganism, and characteristics of the support **(**Suzanne et *al*., 2011). Immobilization of microbial cells on porous carriers cells accumulate mainly due to steric retention, However, in order to use this technology, the immobilized carrier must be cheap and cell immobilization should be achievable with minimal additional cost. In addition, the carrier should preferentially be also renewable considering the nature of intended purpose of its fermentation product.

A natural pozzolana is siliceous or siliceous and aluminous material (Volcanic ash, volcanic tuff, pumicite). It's defined either a raw or calcined natural material that has pozzolanic properties (reacting with Ca $(OH)_2$ in water). The properties of natural pozzolanas vary considerably, depending on their origin, because of the variable proportions of the constituents and the variable mineralogical and physical characteristics of the active materials available in abundance at lower prices it was found to be a very economical and excellent support matrix for attachment of cells. It's should high porosity, high specific pore volume, stable physical properties, non-toxicity and low cost (Alves et *al.,* 1998). This could be promising for achieving large-scale and economical production of lactic acid by immobilized bacteria cells.

Lactococcus lactis subsp lactis is the mesophilic strain most commonly used as starter culture for lactic products. Which consistently yielded highest concentration of exclusively L (+) lactic acid in milk (Boonmee et *al.,* 2003) Furthermore *Lactococcus lactis subsp lactis* is able also to ferment galactose a breakdown product in the fermentation of lactose. It converts directly over 90% of consumed sugar in lactic acid (De Roissart et Luquet, 1994). In the present study, we have developed a process for direct productions of lactic acid from whey permeate using a strain of *Lactococcus lactis subsp lactis* isolated from Algerian goat milk. Conveniently immobilized within pozzolana at high cell density in a packed-bed bioreactor. This study is aimed at producing the highest amount lactic acid from fermentation of whey permeates.

MATERIALS AND METHODS

Carrier

The carrier used in the study was a natural pozzolana (PZ) extracted from Beni-Saf quarry, in the west of Algeria, has the appearance of crushed pumice stone and slag. This material was described by Belas B. et *al*., (2003).

Mineral and Chemical Analysis

X-ray diffraction (XRD) was carried out to determine the mineralogical composition of the crude pozzolana before crushing. The XRD data were collected with Philips PW 3710 X-ray diffractometer with Bragg–Brentano geometry using Ni-filtered Cu K α radiation, operating with the voltage of 30 kV and emission current of 20 mA. The step-scan covered the angular range 2 - 60° (2θ) in steps of $2\theta = 0.02°$

Physical Properties

Physical characteristics of the carrier were determined (apparent volumic mass, absolute volumic mass, specific area Blaine, water absorption, porosity, electric conductivity and pH)

Surface Properties

The surface electronic microscopy observations of pozzolana were conducted on a SEM FED (JEOL-JSM-6301F) using an accelerating voltage of 7 kV and a working distance of 15 mm. SI/ AL ratio indicate the Hydrophobicity of the material

Microorganism

Lactococcus lactis subsp lactis SP1 strain was isolated from goat milk in Algeria. It's a microaerophilic and homofermentative bacteria producing mainly L+ lactic acid. It stored in MRS broth with skim milk at – 80°C. This strain was biochemical identified by duplicate, using API 50 CHL (Biomerieux, 2006)

Microbial Adhesion to Solvents (MATS)

MATS was measured according to the method originally proposed by Rosenberg et *al*., (1980) and recently modified by Bellon-Fontaine et *al*., (1996). Three different solvents were tested in this study: hexadecane, which is an apolar solvent; chloroform, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent (Geertsema et *al*., 1993). The analysis of an eventual bacterial lysis under the action of the three solvents was previously monitored with lactic acid bacteria by phase-contrast microscopy. No deleterious effects were observed (Bellon-Fontaine et *al*., 1996).

Culture Media

Whey Supplemented (WS) Preparation: whey powder was used as follow**:** 65 g/l of powder was solubilized in desionised water, after adjusting the pH to 4.5 with 5N HCl, they were heated at 121°C for 15min to denature the proteins and the precipitates were removed by centrifugation at l0 000 rpm/mn for 15 min The supernatants were adjusted to pH 6.5, sterilized at 121°C for 15 min and used as culture media. Yeast extract (5 g/l) and Mg $SO_4.5H_2O$ (3g/L), are used to supplement the whey. This method was adopted by E Ghaly et *al*., (2003)

Immobilization Technique

Support (30g/l) was added to100 ml ws media (in 250 ml Erlenmeyer contained flask) and inoculated at 1% with a overnight culture of *lactococcus lactis subsp lactis* SP1(OD_{600} = 0.5 approximatively 10^8CFU/ml also cultured in ws media. and mixed (80 rpm/mn) for 24 h at 30°C. before the immobilization process, the pozzolana was subjected to burning (in muffle furnace) at 550°C for 24h, and rinsed with distilled water, dried at a temperature of 105°C for 24h. The amount of adsorbed cells onto the support was computed from weighing the difference between before and after burning, rinsed dried pozzolana carrier as up descripted.

Fermentation Experiment

For the preparation of the fermentation inoculum, 10 ml of ws medium was inoculated with 0.1ml of freshly prepared culture of *lactococcus lactis subsp lactis* SP1 isolate and was incubated for 12 h at 30°C. From this preculture 1 ml was added to 100 ml of ws broth medium, medium after 12 h of incubation at 30°C was used for batch and continuous

fermentation. Free and immobilized cell of *Lactococcus lactis subsp lactis* SP1batch fermentation are performed in serial double jacket 200 ml columns each one containing 100 ml of ws media. For immobilized cells additional carrier at 3% (W/V) was monitoring (at different size 1.25, 4 and 6.25 mm) the inoculum consisted of 1% (V/V). Magnetic agitation at 80 rpm are used (just for homogenization), the solid support particles state on inoxydable grid at the bottom of column, separate the magnetic stirrer. Simples (3 ml) were withdrawn at intervals during 192 h. In Continuous lactic acid production the design of fixed bed bioreactor used is illustrated in figure 1. It was composed of a container (2l) containing 1,5l of sterile ws gently agitated with a magnetic stirrer, pumped into a glass column (5 cm inner diameter and 15 cm length) with a peristaltic pump.

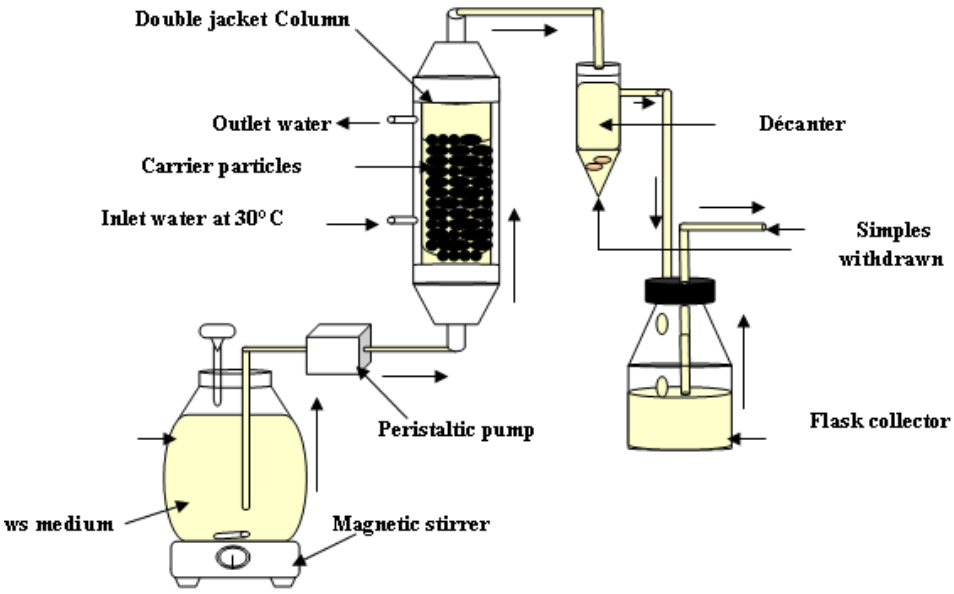

Figure 1: Continuous Bioreactor Design for Lactic Acid Production

The column filled with the corresponding pouzzolana particles size at high over 10cm on an inoxydable grid. The temperature was maintained at 30°C with a thermostatic circulation bath. In the beginning the carrier in bioreactor was immerged with ws medium (pH6.5) inoculated with an overnight culture of *lactococcus lactis subsp lactis* SP1strain at 1% (V/V) (10^8 cfu/ml) without agitation. After 24h the liquid was removed and the carrier washed with sterile distilled water until OD 600nm dropped at the OD value of distilled water and then new ws media was filled in the reactor. The continuous fermentation is conducted with varying the dilution rate D calculated as follow:

$$D = \frac{Q}{V}$$

The dilution rate (*D*), (*Q*): inlet flow, (*V:* volume used in reactor). All experiments are conducted in duplicate and the average is less than 5%.

Analytical Methods

Growth in free cell fermentation was monitored by optical density at 600 nm and converted to dry cell mass using a standard curve. Cells were harvested by centrifugation (10 000 rpm for 10 mn/4°C). Lactose consumption and lactic acid production were determined in the supernatant as descripted above:

Lactose is quantitatively determined by Enzymatic Colorimetric method (EnzyChrom™ Lactose Bioassay Kit ELAC-100) assay uses lactase-coupled reactions in which lactose is cleaved and the resulting galactose forms a colored product. The color intensity at 570 nm is directly proportional to the lactose concentration in the sample.

Lactic acid was mesured by HPLC analysis coupled with an UV variable wavelength detector set to 210 nm. An Aminex HPX-87H ion-exclusion column was eluted with 5 mM sulfuric acid solution at 0.6 ml/min, and the column temperature was maintained at 35 °C.

Statistical Analysis

All experiments were done in triplicate. Standard deviations were calculated and included in the graphical representation of the data.

RESULTS AND DISCUSSIONS

Characteristics of the Carrier Material

The chemical composition data XRD analysis of Algerian natural pozzolana from Beni-saf querry (table 1) is consisting of aluminium silicate material (over than 80% in mass) and the presence of divalent cations like Ca^{2+} and Mg^{2+}.

Table 1: Centesimally Chemical Composition of Algerian Pozzolana from Beni-Saf Querry

SiO_2	AL_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O	SO_3	pH
56,48	17,83	8,92	9,51	4,34	3,0	1,40	0,17	6,5 – 7

The profile showed (figure 2) respectively the prevalence of cordierite ($2MgO.2Al_2O_3.5SiO_2$) and analcime $Al_2O_3.Na_2O.4SiO_2.2H_2O$), small quantities of hematite (Fe_2O_3) and cristobalite (SiO_2).

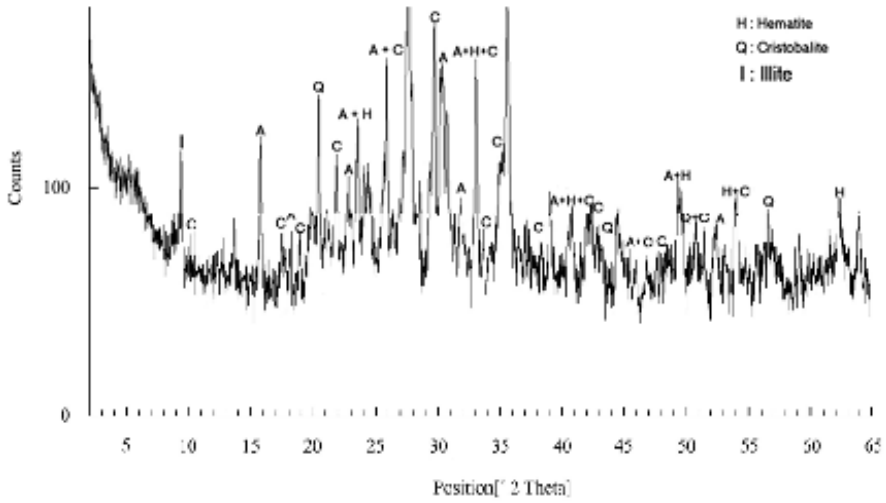

Figure 2: X-Ray Diffraction of the Pozzolana (Cu Kα Radiation, Ni Filtered)

The figure 3 presents the scanning electronic microscopy of the support. It's showed a form of ash and slag and a rough surface. In another hand provide high specific surface and porosity (respectively 3560 cm^2/g and 58, 70 %), chemically stable (pH between 6, 5 – 7 and 0, 02 $\mu S/cm^2$ in electric conductivity). SI/AL index has also been considered important in the adhesion process (SI/AL =2,7 mean a hydrophobic area).

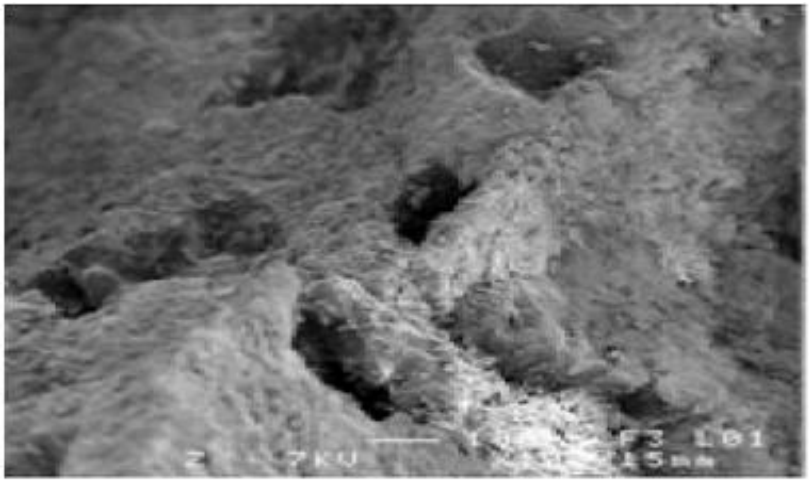

Figure 3: Scanning Electronic Microscopy of Pozzolana Materials

Divalent cations may provide the existence of punctual positive charges on the material surface that can promote the establishment of ionic bridges, resulting in an attractive interaction. (Pereira M et *al*., 2000). Many studies show that the surface hydrophobicity of the mineral carriers is more important for colonization by the anaerobic consortium than the surface charge (figure 3,4 and table 2). In fact, the biomass retention capacity correlates linearly with the degree of surface hydrophobicity biomass retention capacity (Pereira MA et *al*., 2000, Oliveira R et *al.*, 2001).

Figure 4: Pozzolana Materials under Binoculare Loope (x10)

Actually, the specific surface area of the carrier is important to promote biological growth, allow maximum transfer rate and trap suspended solids this leads to high concentrations of attached biomass and hence much smaller reactor (Oliveira R et *al*., 2001).

Table 2: Physical Proprieties of Pozzolana

Physical Characteristics		
Apparent volumic mass	g/cm^3	0,98
Absolute volumic mass	g/cm^3	2,75
Specific area Blaine	cm^2/g	3560
Water absorption	%	58,70
Porosity	%	57,10
Electric conductivity Ce	$\mu S/cm^2$	0,02
Hydrophobicity	SI /AL Ratio	2.7

Microorganism Characteristics

The MATS results obtained for *Lactococcus lactis subsp* SP1grown at 30°C in Eliker broth and supplemented whey are shown in Table 3. Regardless of the medium used, the affinity of *Lactococcus lactis subsp lactis SP1*was always higher with hexadecane (non polar solvent) indicates that the strain is strongly hydrophobic (> 80 per cent). The weak affinity of the strain for ethyl acetate and Chloroform solvents in comparison to the associated non polar solvent revealed a weak electron-accepting or donating nature of the strain. This difference in affinity is due to Lewis acid-base interactions between microorganisms and the hydrocarbon (M.H. Ly et *al*., 2008). In the same way no differences are noted in change cultivation medium.

Table 3: Affinities of *Lactococcus lactis subsp lactis* Cells for the Three Solvents Used in the MATS Analysis after Growth in Either Eliker Broth or WS Medium at 30°C

Solvent	Hexadecane	Chloroform	Ethyl Acetate
Eliker	90 ± 5	6 ± 1	2 ± 1
Ws	92 ± 6	8 ±,2	4 ± 1

The measurement of cell surface hydrophobicity can be considered as an indicator of the ability of cells to adhere to the solid surfaces or interfaces. These properties have been studied to understand the first step of the adhesion of bacteria which is the cause of biofilms formation (Donlan, 2002; Strevett and Chen, 2003). The surface of lactic acid bacteria strains studied in literature is rather hydrophilic (Boonaert and Rouxhet, 2000; Pelletier et *al*., 1997). Several studies have investigated the composition, structure and forces involved in bacterial and results confirm that hydrophobicity index might be dependent on the carbon source used as energy substrate the highest hydrophobic percentage demonstrates that this microorganism presented important superficial characteristics, implicating contact between the bacterial cell membrane and the interacting surfaces. According to these authors, the chemical groups of proteins, polysaccharides, peptidoglycans and lipoteichoic acids at the cell surface are responsible for its physicochemical properties. The bacterial surface charge results from the dissociation or protonation of three main ionizable groups, the phosphate-group of (lipoteichoic acids and the carboxyland amino-groups of proteins, which depend on pH.

Kinetic of Cells Immobilization

Experiments were conducted in three stages. At the first stage, analyses were carried out for the adsorption capacity of the porous carrier for immobilization of bacteria cells with three particle size. The analyses were aimed to identifying a relationship that determines the effect of selected particles on immobilization process activity compared to free cells. The second stage of the research focused on lactic acid batch fermentations with immobilized and free cells. The third stage is focused on continuous fermentation with immobilized cells bed filled with optimal particles size. Comparative growth of free and immobilized cells (with carrier at different particle size in batch culture showed in figure 5.

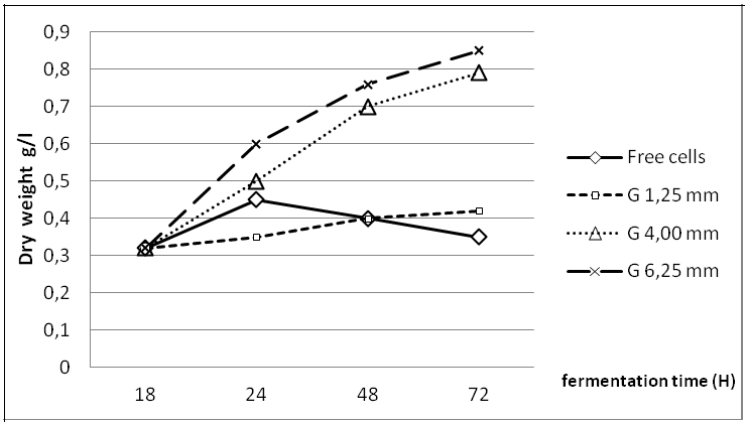

Figure 5: Attachment Cells of *Lactococcus lactis subsp lactis* SP1Cultured at 30°C in Eliker Broth Filled with Different Granulometric Particle of Pozzolana Expressed as Dry Weight Biomass Determined by Weight Loss after Heating at 550°C Compared to Free Cell Culture Dry Weight

Batch Fermentation

In batch fermentation; data shown that free cells biomass decrease rapidly after twenty four hours of culture (reaching the stationery phase). But the immobilized cells gradual increase in specific growth in a relationship with size particle carrier. The highest in growth is showed with the biggest diameter of particles (6,25 mm). The Biomass adsorbed expressed as g of dry weight per g of support is respectively 0,6 ± 0,003 at 6,25 mm, 0,5 ± 0,007 at 4mm and 0,35 ± 0,0032 at 1,25 mm. These results mean that pozzolana offer good conditions for improved maintenance of high cell density. The support has a lot of pore with suitable dimension for immobilizing lactococcus. The adsorption cells of *lactococcus lactis subsp lactis* SP1 in beds made possible the increasing of lactic acid production as a result of the increasing of the biomass (figure 6 and 7). The lactic acid concentration was increased by 40 %, compared with the fermentation with free cells. The fermentation with particle size bed 6,25 mm is the most efficiency. Lactose consumption in the time course when 45,0± O,65 g/l lactose concentration used is respectively:

- 21% in 1,25 mm particles bed
- 38% in 4 mm particles bed
- 77% in 6,25 particles bed

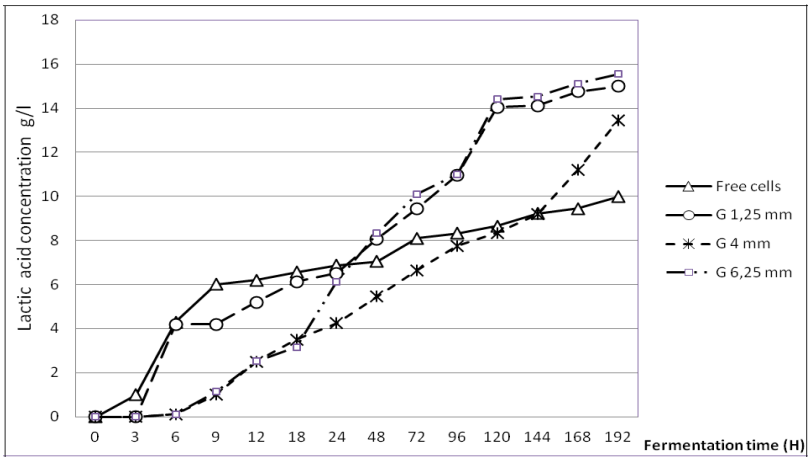

Figure 6: Lactic Acid Production (g/l) with Free and Adsorbed Cells of Strain of *Lactococcus lactis subsp lactis* SP1 onto Pozzolana Carrier Cultivated at 30°C in WS Medium

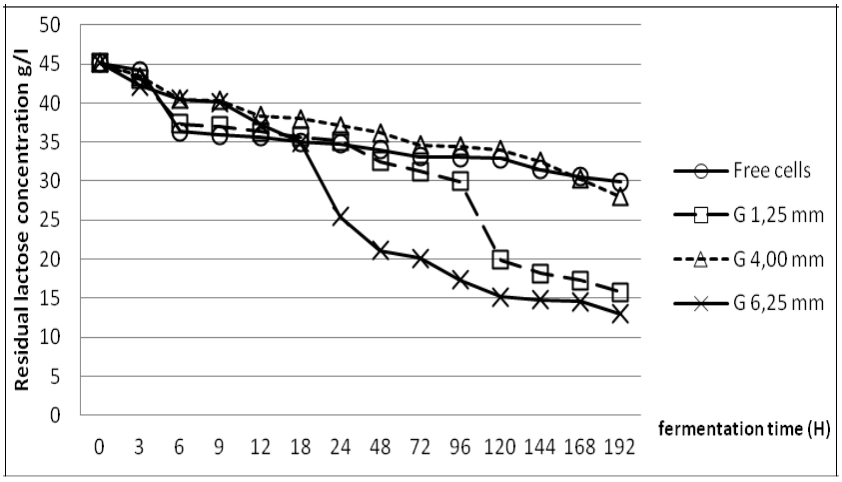

Figure 7: Residual Lactose (g/l) with Free and Adsorbed Cells of Strain *Lactococcus lactis subsp lactis* SP1 onto Pozzolana Carrier Cultivated at 30°C in WS Medium

Continuous Production of Lactic Acid

Continuous fermentation experiments were conducted to examine the potential of this system for industrial use, and to determine conversion rates, acid yields and productivities under long-term sustained operation conditions. A fresh immobilized cell reactor with the bacterium *Lactococcus lactis subsp lactis* SP1 was prepared and fed with ws production medium in bed filled with 6, 25 mm particles size. Four dilution rates are used corresponding to respectively 0, 21, 0,26, 0,31, 0,36, 0,41 and 0,46 h^{-1}. Figure 8,9 and 10 shows the results from continuous operation over a period of 20 days with an initial lactose concentration of 45.0 ± 0,65g/L. The results obtained presented show in generally goods yield in all dilution rates and similar qualitative curves according to the duration of the fermentation. In all cases the steady state is reached after 12 days. Maintained and stability of the steady state are reached with all dilution rates. In general, all of the options tested are suitable for reaching high lactic acid productivities within a period of up to 12– to over 20 days.

The lactose conversion rate was ranged from 88,88 to 94,75%, the acid yields ranged from 10,26 ± 0,45 to 21,80 ± 0,34 and the acid productivities were ranged from 14,36 ± 0,33 to 30,57± 0,32 g/l.h respectively from dilution rates (D) 0.21/h to 0.46/h. The greatest efficiency in lactic acid production is obtained at 0,46 h^{-1} dilution rate. In without controlled pH and at high dilutions rates conditions, lactic acid production is not growth associated. It was observed that as the dilution rates increased the dry weight of biomass decreased. In steady state the highest level of biomass is reached at 0, 21/h (over 2,5 g per g of carrier) and the lowest is at 0,46/h (less then 0,5 g/g). The number of immobilized cells depended on the type of carrier which is in agreement with a previously observation that the loading rate of immobilized cells decrease with the increase of particle size of material. As higher the external surface area, the higher is the immobilization capacity. (Harendranath et *al*., 1996). Adsorbed cells are in direct contact with the surrounding environment and hence subject to any forces of shear or attrition which may result from the relative motion of particles and fluid. It is therefore likely that some cells will become detached and enter the bulk fluid phase.

It is also difficult to control or even determine the depth of the attached biofilm. In another hand it is reported that in high density cells reactors uncoupling between growth and lactic acid production was observed, when the maximum population was reached. The enhanced lactic acid production under immobilized conditions may be attributed to improved

buffering activity of fermentation medium. Such increased production profile as well as the associated metabolic affects on cell growth and subsequent product formation was well documented in literature.

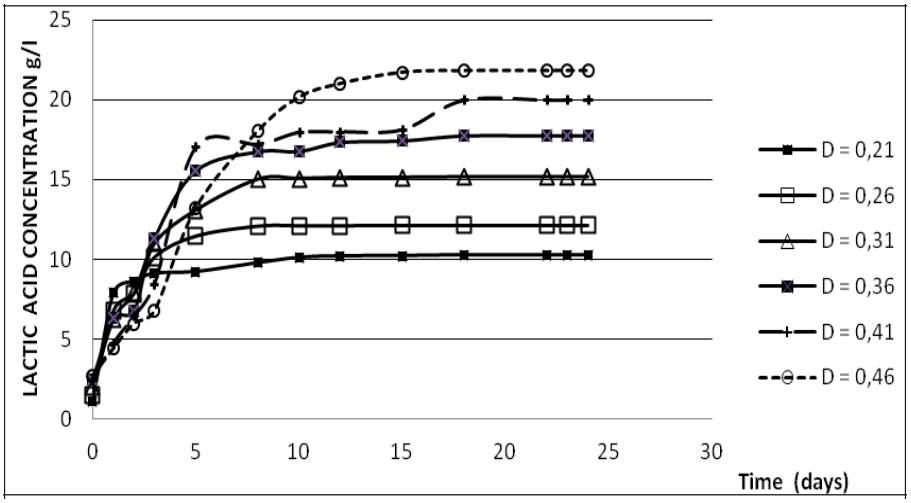

Figure 8: Continuous Lactic Acid Production (g/l) at Different Dilution Rates and Time in WS Medium at 30°C

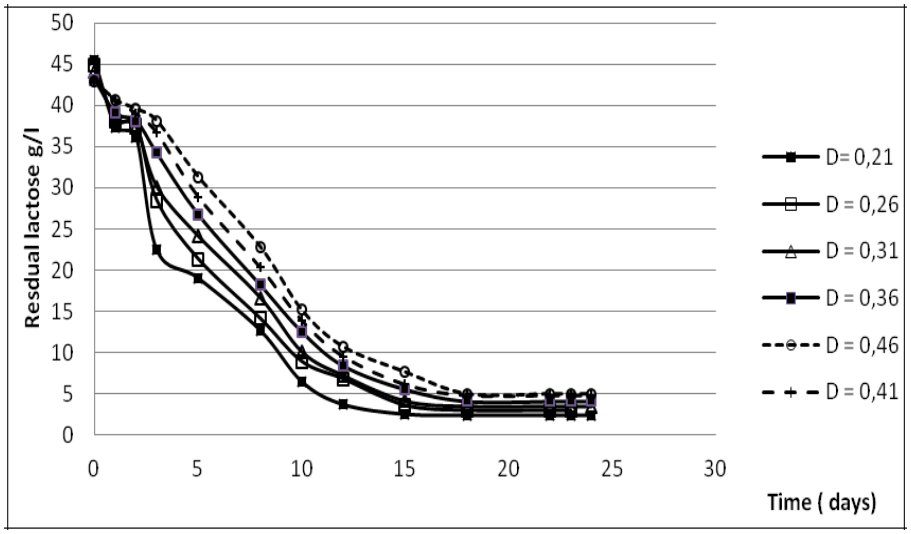

Figure 9: Residual Lactose at Different Dilution Rates and Time of Continuous Lactic Acid Production

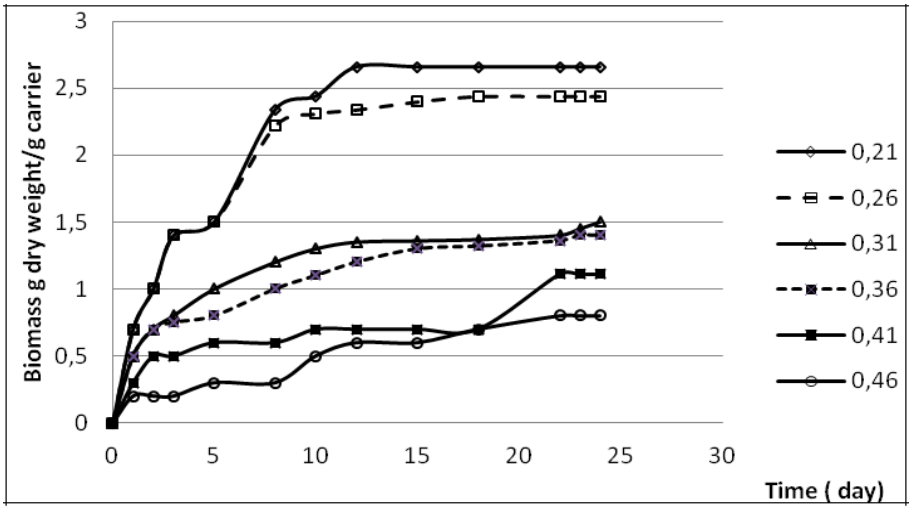

Figure 10: Dry Weight of Biomass at Different Dilution Rates and Time Continuous of Lactic Acid Production

Table 4 summarizes some of the relevant results obtained in continuous lactic acid setup with immobilized cells of the strain *Lactococcus lactis subsp lactis* SP1. The correlation matrix revel a very good relationship between dilution rate productivity and lactic acid production ($0,99 \leq R^2 \leq 1$).

Table 4: Relevant Results of Lactic Acid Production, Residual Lactose Conversion and Productivity at Different Dilution Rates in Continuous Mode

Dilution Rate (h^{-1})	Lactic Acid g/l	Residual Lactose g/l	Conversion %	Productivity $g.l^{-1}.h^{-1}$
0,21	10,26	2,36	94,75	14,36
0,26	12,72	2,93	93,49	17,81
0,31	15,16	3,48	92,26	21,22
0,36	17,6	4,11	90,88	22,71
0,41	19,48	4,80	89,33	27,32
0,46	21,8	5,00	88,88	30,57

The strain is very stable on the variety of experiment conditions examined. Several studies have shown that, cells produced with immobilized cell technology exhibited a change in growth, morphology and physiology characteristics compared with cells produced during conventional free cultures (Doleyres, 2003). Differences in cell physiology during batch cultures without pH control and continuous cultures with free and immobilized *Lc. lactis subsp lactis SP1*were depending on the culture mode. The redox states, enzymatic pool and intracellular pH differed for immobilized and free-cell cultures and genetic expression (Cachon et *al*., 1998). All of the investigations have been undertaken with respect to both the downstream steps and the economy of the whole process and, therefore, can also be used for technical lactate production. Further investigations are necessary to develop large-scale lactate production with this system based on a low-cost medium optimized in such a manner

CONCLUSIONS

Evaluation of the system for lactic acid production in reactors using immobilized *Lactococcus lactis subsp lactis* SP1 bacteria by natural adsorption onto pouzzolana showed that acid productivity was better in continuous system than in batch culture. The lactic acid fermentation is greatly influenced by the particle size of pozzolana. In without controlled pH and at high dilutions rates conditions lactic acid production is not growth associated. The results suggested also that in steady state a stability of continuous production of L+ lactic acid is obtained in appreciable final concentration, the high conversion rates and acid productivity values indicate good potential for the use of this process in the continuous treatment of whey effluents both to produce technical or pure lactic acid easily coupled with efficient membrane purification device.

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